American Journal of Immunology

Clinical Procedures

The Ruggiero-Klinghardt (RK) Protocol for the Diagnosis and Treatment of Chronic Conditions with Particular Focus on Lyme Disease

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Article history
Received: 20-02-2017
Revised: 28-02-2017
Accepted: 08-03-2017

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Abstract: Here we describe the Ruggiero-Klinghardt (RK) Protocol that is based on integration of Autonomic Response Testing (ART) with diagnostic ultrasonography and on application of therapeutic ultrasounds; the latter are used as a provocation tool and as an instrument to optimize drug uptake and utilization in specific areas of the body. This protocol consists of a precise sequence of diagnostic and therapeutic procedures with the ultimate goal of improving sensitivity and specificity of diagnosis at the same time evaluating and optimizing efficacy of treatments in chronic conditions including, but not limited to, persistent Lyme disease. The RK Protocol represents a paradigm shift in diagnostics and therapeutics: Thus, compartmentalized microbes, transformed cells, toxins and metabolites could be detected using a safe and non-invasive method. In addition, the RK Protocol allows optimization of efficacy of drugs and other therapeutic interventions. Although the RK Protocol was initially developed for persistent Lyme disease, it shows significant potential in conditions ranging from cancer to neurodegenerative diseases and autism. In oncology, the RK Protocol may serve to facilitate early diagnosis and to increase sensitivity of cancer cells to the killing effects of a variety of remedies ranging from conventional radio- and chemotherapy to more recent forms of immunotherapy. Thus, the 1st goal of the RK Protocol is diagnostic: That is, to make pathogens, toxins, transformed cells and cells infected by viruses that are inaccessible to conventional diagnostic and therapeutic tools, "visible" to the therapist who can detect them with laboratory methods and deal with them with appropriate interventions; and also to make them "visible" to the immune system that can fight them in a physiological manner. The 2nd goal is to optimize drug uptake and utilization in the organs and tissues studied and targeted with these procedures.

Keywords: Lyme, Ultrasound, Autonomic Response Testing, Immune System, Imaging, Brain

Introduction

The infectious disease known as Lyme borreliosis, or Lyme disease, is the most common infection due to tick bites and sometimes also to other stinging insects in the Northern Hemisphere. Although estimates vary and it is likely that the number of cases worldwide is much higher, there is general consensus that the disease affects hundreds of thousands of individuals a year in North America, Europe and northern Asia with incidence of the infection on the rise (Shapiro, 2014; Gingrich *et al*., 2001). The designation "Lyme disease" derives from the small New England villages of Lyme and of Old Lyme where the arthritic manifestations of the disease were first described in 1975 as "a rather random clustering of several cases of juvenile chronic arthritis" (Burmester, 1993).

The etiologic infectious agent, *Borrelia burgdorferi* was identified by Wilhelm "Willy" Burgdorfer it in 1981 and, since the original observation limited to arthritis, the clinical spectrum of the infection now includes a variety of clinical conditions such as erythema migrans,

acrodermatitis chronica atrophicans, lymphadenosis benigna cutis, arthritis, myocarditis, neuroborreliosis (meningo-encephalitis, meningo-radiculitis, meningitis), myositis and various ocular and skin disorders (Burgdorfer, 1991; Chomel, 2015). Interestingly, as knowledge of the disease deepened, the increase in the number of clinical manifestations of the infection was paralleled by the increase in the number of spirochete bacteria belonging to the genus Borrelia associated with the disease; *Borrelia burgdorferi sensu stricto* is found in the Americas whereas *Borrelia afzelii* and *Borrelia garinii*, in addition to *Borrelia burgdorferi*, are observed in Europe and Asia (Chomel, 2015). In a sort of an exponential increase in complexity, it was later discovered that the ticks responsible for transmitting the *Borreliae* that are *Ixodes scapularis* and *Ixodes pacificus*, also have the potential to transmit an increasingly expanding list of other pathogenic microbes that include bacteria, viruses and parasites such as *Anaplasma phagocytophilum*, *Babesia microti*, deer tick (Powassan) virus, *Borrelia miyamotoi* and the *Ehrlichia muris*-like organism (Caulfield and Pritt, 2015). Obviously, the presence of coinfections renders the treatment and the diagnosis rather difficult and contributes to patient morbidity and mortality as well as to the appearances of the so called "post-treatment Lyme disease" also known as "chronic Lyme" or, as we prefer to denominate it in this study, "persistent Lyme disease". Thus, persistence of *Borrelia burgordferi* in tissues after efficient antibiotic treatment has been demonstrated in a variety of experimental models that include primates, but there is currently no affordable, non-invasive, method to detect specific persistent microbes (or their metabolites) in vital organs such as the brain, spinal chord or heart (Straubinger *et al*., 1997; 2000; Hodzic *et al*., 2008; Yrjänäinen *et al*., 2010; Embers *et al*., 2012).

Persistent Lyme is a diagnosis that is given to patients with prolonged, unexplained symptoms, that may be severe, occurring after efficient antibiotic treatment. Such symptoms are most often non-specific and include neurological symptoms such as chronic fatigue, difficulty to sleep, inability to focus, irritability, depression, headache and dizziness; gastrointestinal symptoms such as abdominal pain, nausea and diarrhea; symptoms suggestive for local or systemic inflammation such as pharyngodynia, enlarged lymphnodes, muscle rigidity, myalgia and arthralgia (Feder *et al*., 2007). The non-specificity of these symptoms and the absence of reliable laboratory tests has led to questioning the very existence of a persistent Lyme disease since it is claimed that there is no strict evidence that the chronic post-treatment symptoms are attributable to ongoing infection with *Borrelia burgdorferi* or with any other identified organism (Feder *et al*., 2007; Halperin, 2015). However, despite the controversies surrounding the existence, the definition or the nature of persistent Lyme, it is indisputable that this condition leads to significant sequelae that dramatically decrease the quality of life of affected patients. Thus, a recent study from the John Hopkins School of Medicine describes the physical and social limitations associated with the condition that lead to fundamental changes in the way of living. In this study, the Authors evidence the disease-specific factors that contribute to symptom and illness invisibility and the pervasive medical uncertainty regarding persistent Lyme that promotes an increased sense of personal responsibility for care. The Authors conclude that "similar to other contested or medically unexplained syndromes, our findings suggest that the social sequelae of post-treatment Lyme disease/chronic Lyme can be equally protracted as the physical effects of this illness" (Rebman *et al*., 2015).

The main reason for the contested status of persistent Lyme as a fully recognized disease mainly resides in the objective difficulty to assess the persistence of *Borrelia*, or other pathogens responsible for coinfections, after efficient antibiotic treatment. However, persistence of *Borrelia burgordferi* in tissues after antibiotic treatment has been demonstrated in a variety of experimental models, which include primates, that were infected with *Borrelia burgdorferi* and then received aggressive and successful antibiotic treatment for 4-6 months (Straubinger *et al*., 1997; 2000; Hodzic *et al*., 2008; Yrjänäinen *et al*., 2010; Embers *et al*., 2012).

The latter study demonstrates that the pathogen is able to resist antibiotic treatment in primates, even though the *Borrelia* in itself is not known to possess resistance mechanisms and it is susceptible to common antibiotics such as doxycycline and ceftriaxone *in vitro*. Therefore, mechanisms other than classic antibiotic resistance have been hypothesized to explain the persistence of the bacteria and these include the formation of drug-tolerant persister cells (Sharma *et al*., 2015), cell wall deficient forms and biofilm residing forms, as well as the generation of cystic forms of *Borrelia* with low metabolic activity that enables the spirochete to survive in a hostile environment until conditions are favorable to proliferate again (Murgia and Cinco, 2004).

The controversies and the difficulties associated with the diagnosis and treatment of persistent Lyme could be resolved by the existence of reliable and reproducible tests that were able to ascribe the symptoms to chronic infections rather than to a wide number of other conditions that may present themselves with similar or identical symptoms (Eshoo *et al*., 2013; Curcio *et al*., 2016).

At Sophia Health Institute, we use a manual biofeedback technique, denominated Autonomic Response Testing (ART) that has the goal of assessing

the presence and/or the persistence of spirochete and other infectious agents that may be associated with persistent Lyme disease. ART represents an evolution and an expansion of the technique that was originally proposed by Omura (1981) and subsequently validated in a number of studies including two randomized-order blinded studies registered as a clinical trial (Jacobs *et al*., 1984; Jensen *et al*., 2016). In a paper published in 2016, ART developed at the Sophia Health Institute was utilized by independent researchers in the diagnosis and treatment of painful scars. In this study, the Authors state that "In our experience, ART produces useful and consistent information most of the time" (Chung and LaRiccia, 2016). The validity of ART was further confirmed in another independent investigation focused on breast cancer (Chung and LaRiccia, 2017).

In addition to ART, at Sophia Health Institute, we investigate the presence of pathogens in urine samples of patients using Polymerase Chain Reaction (PCR) in order to identify genes specific for microbes known to be associated with persistent Lyme. However, we noticed quite often that, while ART suggested the presence of pathogens, such a presence was not confirmed by the PCR-based DNA test performed on urine samples. Other researchers have noticed the low sensitivity of the urine DNA test when it is performed without the provocation method that will be described in the following sections of this study. However, serendipitous observation at Sophia Health Institute suggested that the results of the urine DNA test matched more closely the results of ART when urine samples were collected after the patient had received a deep tissue massage with focus on the symptomatic body regions; massage that had been performed to relieve tension or for other reasons. Based on these observations and in search for a reliable method to improve the sensitivity of the urine DNA test, we decided to apply pulsed therapeutic ultrasounds focused on critical areas to mimic or improve the effects of deep tissue massage. This approach is based on the widely recognized feature of ultrasounds to transmit waves of compression and relaxation in biological tissues with resulting changes in organ, cellular and molecular structures that can be exploited in the context of therapy (Leinenga *et al*., 2016). Thus, we have recently demonstrated that non-thermal ultrasounds induce quasi-instantaneous changes in human neurons and murine microglial cells in vitro with results that are consistent with the observed effects of ultrasounds on mental states (Cosentino *et al*., 2015; Bocchi *et al*., 2015; Hameroff *et al*., 2013).

Here we describe an original protocol denominated Ruggiero-Klinghardt (RK) Protocol that is based on ART and on a precise sequence of diagnostic and therapeutic ultrasounds for the accurate and reliable diagnosis and treatment of persistent Lyme disease and other chronic conditions.

Materials and Methods

Ultrasound Systems

For diagnostic ultrasonography, we used a portable MicroMaxx ultrasound system manufactured by Sonosite, Bothell WA, USA, with color-doppler application and with a linear (L38e) and a convex (C60e) transducer. This system is approved for many applications including cephalic (brain) imaging and has the same features of the system that one of us (M.R.) had previously used to characterize the lesions in the brains of autistic children (Bradstreet *et al*., 2014); in this latter study, the safety of the procedure is thoroughly described. In order to avoid any bias, the images were not exported and pictures of the screen of the ultrasound system were taken with the camera of a common smartphone. This procedure was chosen in order to avoid the possibility of editing the images, that are presented here exactly as they were produced; this procedure, however, inevitably leads to poor quality images since these were taken at the bedside without any consideration for professional photo-shooting. An example can be observed in Fig. 4, where portions of the screen of the ultrasound system can be observed. The pictures of the images were then copied in a Power Point file in order to add captions, arrows or circles that are useful to show the anatomical structures that were studied and their alterations. The application of therapeutic ultrasounds, that is an essential part of the RK Protocol, was performed using an Intelect TranSport Ultrasound Therapy device (Chattanooga Medical Supplies Inc., Chattanooga TN, USA). This system enables pulsed and continuous operation (10, 20, 50 and 100%) on 1 and 3.3 MHz frequencies using a 5 cm² sound head applicator.

Urine DNA Test

Urine samples were collected before and after application of therapeutic ultrasounds at Sophia Health Institute according to the protocol summarized in Table 1 and following the recommendations of the company performing the DNA test. The test was performed by DNA Connexions, Colorado Springs CO, USA. The Lyme panel tests for 4 different genes that are found in *Borrelia burgdorferi* and 8 common Lyme disease co-infectors including *Babesia microti*, *Babesia divergens*, *Babesia duncani*, *Bartonella bacilliformis*, *Bartonella henselae*, *Bartonella quintanta*, *Borrelia miyamotoi*, *Borrelia recurrentis*, *Ehrlichia chaffensis* and *Anaplasma phagocytophilum*. According to the company and consistent with common knowledge, a positive PCR-based Lyme test indicates the presence of DNA from *Borrelia burgdorferi* and/or other co-infectors.

Table 1. The Ruggiero-Klinghardt (RK) Protocol steps of diagnostic and therapeutic procedures

Step 1.	First comprehensive Autonomic Response Testing (ART).
Step 2.	Total-body diagnostic ultrasonography that has the role of further refining the diagnostic hypotheses put forward by ART.
Step 3.	Application of therapeutic ultrasounds with particular focus on the areas identified as "abnormal" with the previous steps.
Step 4.	Second ART performed after application of therapeutic ultrasounds: Comparison of the results with those obtained with the first ART of Step 1.
Step 5.	Collection of midstream urine in a sterile container that is then shipped to the laboratory. Alternatively, the presence of microbes, toxins, circulating cancer cells or other pathogenic noxae is investigated in other biological matrixes such as stools, blood or serum or breath that are appropriately collected.
Step 6.	Pharmacological treatment with remedies specific for the identified pathogens.
Step 7.	Daily application of therapeutic ultrasounds targeted to the areas identified with the previous steps.
Follow-up at 3-4 month intervals to evaluate the effectiveness of treatment and to assess the treatment end-point.	

A negative result, however, does not prove that a patient is not infected with a tick borne infection, rather it indicates the absence of detectable DNA pertaining to microbes associated with Lyme and/or other tick borne co-infections in that particular urine sample. Stage of infection, timing of courses of antibiotics and persistence of latent microbes in reservoirs or sanctuaries, are only some of the factors that may affect the detectability of the DNA of spirochetes in urine samples.

Autonomic Response Testing (ART)

ART, invented and developed by one of us (D.K.), represents an evolution and an expansion of the test originally described by Omura (1981).

At variance with the test proposed by Omura, ART takes into account the entirety of the autonomic response and not only the strength or the resistance of muscles. This is particularly important in the study of parasympathetic activity and in the evaluation of the balance between sympathetic and parasympathetic activities. A core principle of the test relies on the "Resonance Phenomenon Between Identical Substances". Thus, a culture of a particular pathogen is used to non-invasively detect the presence of this very pathogen in a particular body region or organ. A description of ART can be found in a recently published peer-reviewed study (Chung and LaRiccia, 2016). In the present study, as *per* the RK Protocol, ART was performed two times, *i.e.,* before and after application of therapeutic ultrasounds as summarized in Table 1; the results of the two tests were then compared and recorded. Since interpretation of ART can provide information on the potential responsiveness of the patient to different therapeutic approaches and since the involvement of the immune system in persistent Lyme is widely acknowledged, we used an emulsion of chondroitin sulfate, vitamin D_3 and oleic acid endowed with immune modulating properties as positive control (dr. reinwald healthcare, Schwarzenbruck, Germany). The characteristics of this emulsion and its potential use in integrative immunotherapy have been recently described (Schwalb *et al*., 2016).

Results

Description of the RK Protocol

Step 1

After having collected a careful anamnesis and critically reviewed clinical records, laboratory exams and radiological images, the patient is invited to empty her/his bladder before performing the first step of the RK Protocol that consists in ART which is performed by the therapist with the help of an assistant; the sequence of steps of the RK Protocol is summarized in Table 1. ART provides the initial information that is useful to restrict the spectrum of diagnostic hypotheses and to identify the organs or the areas of the body that need further investigation.

Step 2

Then, as the second step of the protocol, a diagnostic total body ultrasonography is performed with particular focus on those areas that have tested positive with ART.

The following examples elucidate the synergy between ART and ultrasonography in diagnostics. Figure 1 shows the ultrasonographic appearance of the submandibular salivary gland of a subject who had tested positive with ART in that area. The inhomogeneous appearance of the gland with irregular hypoechoic areas is indicative of a diffuse inflammatory process whose origin (viral or autoimmune) requires further investigation. Figure 2, shows an enlarged and possibly inflamed, deep cervical node in a subject with symptoms of neuroborreliosis who had tested positive with ART. The blood vessels in the hilum of the node are clearly visible at the echo-color-doppler and their appearance is consistent with a condition of hyper-afflux. It is worth noticing that inflammation of the deep cervical nodes may be associated with impaired lymphatic drainage from the brain lymphatic system (also known as "glymphatic system") with consequent stagnation of lymph in the brain and accumulation of metabolites and neurotoxins in addition to potential disruption of

the brain microbiota. These events may be associated with, if not responsible for, some of the symptoms of neuroborreliosis, autism and other neurological diseases (Bradstreet *et al*., 2014; Ruggiero, 2016). Figure 3, shows the appearance of the vagus nerve in a subject who had tested positive with ART for parasympathetic imbalance. In this transversal projection, the vagus nerve appears as a small triangular structure located posteriorly inside the carotid sheath between the common carotid artery and the internal jugular vein; it shows an internal honeycomb structure. In this subject, the epineurium appears as a thickened hyperechoic ring surrounding the nerve. Figure 4, shows the appearance of the thyroid in longitudinal projection in a subject who had tested positive with ART for parathyroid involvement. The arrow indicates a roundish area that protrudes from the posterior margin of the thyroid and could be interpreted as an enlarged parathyroid gland. Please notice that the solid arrow was inserted during the ultrasound examination whereas the dotted circle was inserted during the preparation of the figure, for the sake of clarity, using a Power Point program.

Step 3

After having performed the diagnostic total body ultrasonography, the third step of the RK Protocol is then performed; therapeutic ultrasounds are directed toward those organs or areas of the body where clinical suspicion of inflammation, proliferative, degenerative or infectious diseases is present. The rationale for this procedure is to exploit the mechanical effects of ultrasounds in human tissues with the goal of mobilizing pathogens, toxins, transformed cells, cells infected by viruses, or cells of the immune system carrying pathogens, toxicants or antigens of transformed cells, so that they may be identified by the second ART and by specific tests such as the urine DNA test. In other words, the goal of application of therapeutic ultrasounds is to force the exit from reservoirs or sanctuaries where pathogens or transformed cells are invisible to the immune system and to the therapist performing ART or using laboratory methods and are protected from therapeutic intervention (Cory *et al*., 2013). Choice of the pulsed sequence and frequency depends on the location of the organ to be treated.

For example, when treating the spleen, a major organ of the immune system, we choose a pulsed sequence indicated as 50% and a frequency of 1 MHz. First the spleen is identified by diagnostic ultrasonography (Fig. 5) and then it is treated for 3 min, slowly moving the sound head applicator so to send the ultrasound waves to most areas of the organ. The effectiveness of application of therapeutic ultrasounds is then assessed by studying the blood flow in the spleen after the treatment, using for this purpose the diagnostic echo-color-doppler technique. As shown in Fig. 6, immediately after application of therapeutic ultrasounds, a significant increase in blood flow can be observed at the diagnostic ultrasonography. We have previously demonstrated that such an increase corresponds to the activation of cells of the immune system inside the spleen, with particular reference to macrophages (Ruggiero *et al*., 2014). Thus, the mechanical waves of compression and relaxation of therapeutic ultrasounds "squeeze" the organ, presumably at the level of the microscopic anatomy of spleen as well as at the level of cellular and molecular structures such as the proteins of the cytoskeleton (Hameroff *et al*., 2013).

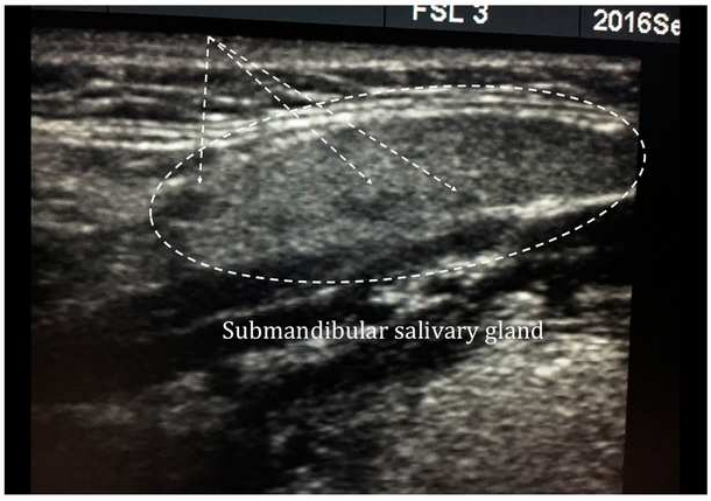


Fig. 1. Ultrasonographic appearance of the submandibular salivary gland

DOI: 10.3844/ajisp.2017.114.126

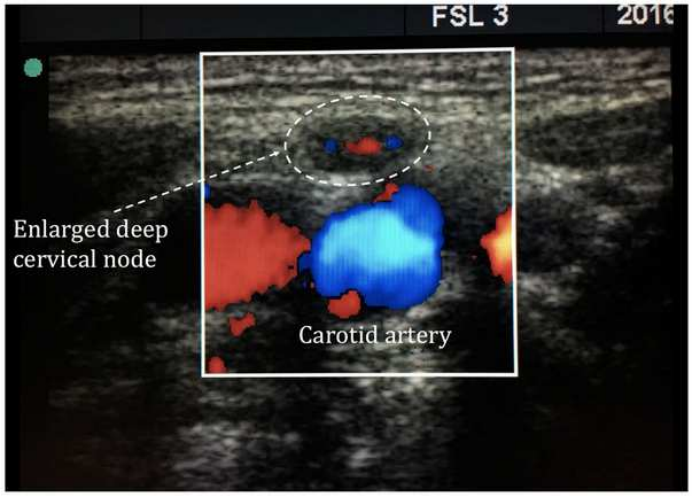


Fig. 2. Ultrasonographic appearance of a deep cervical node

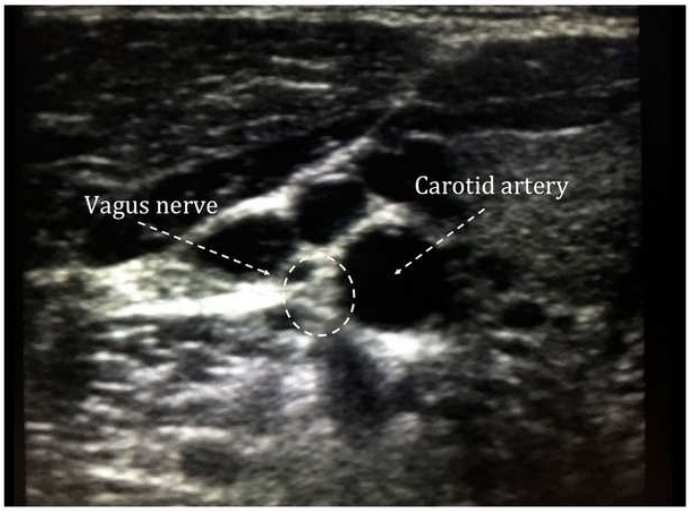


Fig. 3. Ultrasonographic appearance of the vagus nerve

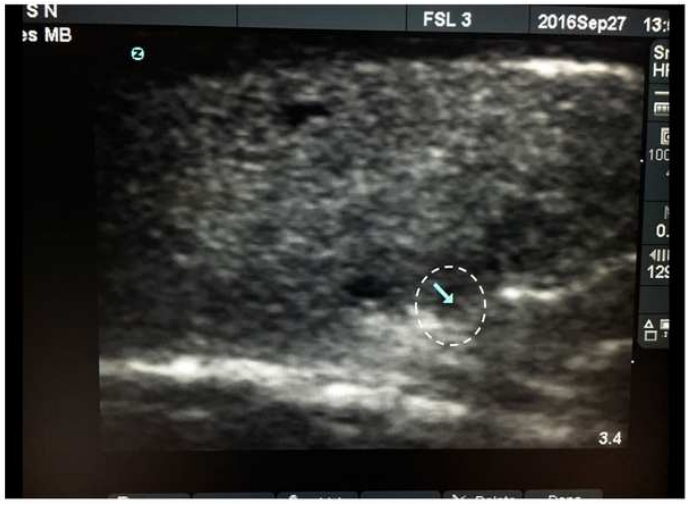


Fig. 4. Ultrasonographic appearance of the thyroid

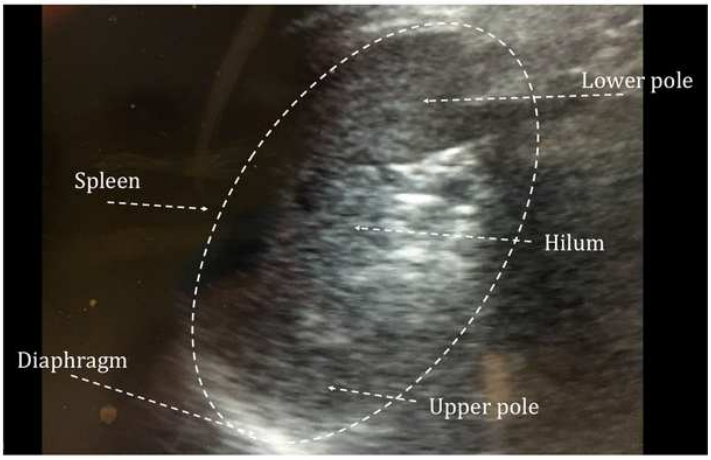


Fig. 5. Ultrasonographic appearance of the spleen before therapeutic ultrasound treatment

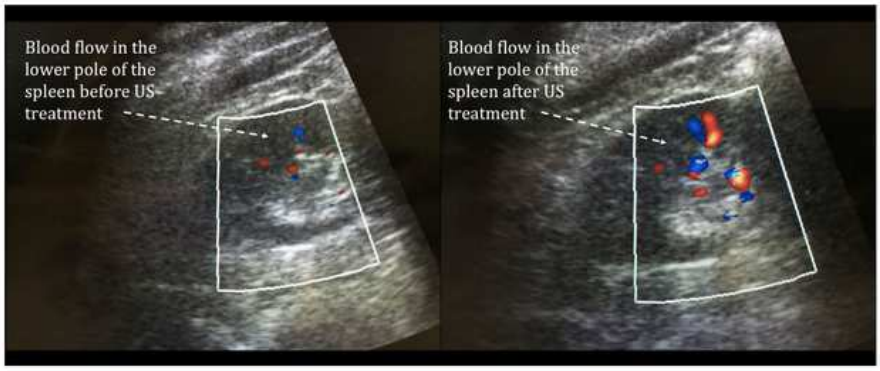


Fig. 6. Blood flow in the lower pole of the spleen before and after application of therapeutic ultrasounds

We hypothesize that mobilization of pathogens or toxins from sanctuaries or reservoirs is followed by their elimination through the urine and this phenomenon should optimize the sensitivity of the urine DNA test. In addition, we have observed that the sensitivity and the specificity of ART significantly increase after application of therapeutic ultrasounds, thus improving the intra- and inter-operator reproducibility of the technique. Furthermore, as we have observed using the immune modulating emulsion mentioned in the Materials and Methods, application of therapeutic ultrasounds to a specific organ significantly improves the sensitivity of the organ to the remedy. Such an effect did not come as a surprise since it is known that ultrasounds have the potential to increase the effectiveness of a number of drugs and remedies by favoring their cellular uptake and overcoming the barriers that prevent delivery of drugs to specific lesions (Bui *et al*., 2017).

We observed that the biological effects of application of therapeutic ultrasounds were particularly remarkable when the technique was applied to the brain, with particular reference to the temporal lobes. We and others have previously demonstrated that ultrasounds could be directed toward specific areas of the brain using carefully selected acoustic windows such as the temporal squama (Ruggiero *et al*., 2013; Hameroff *et al*., 2013; Bradstreet *et al*., 2014).

More recently, we have demonstrated that ultrasounds elicit cellular and molecular responses in neurons and glial cells that are consistent with their effects on mental states and can be exploited in the context of therapy (Cosentino *et al*., 2015; Bocchi *et al*., 2015; Hameroff *et al*., 2013; Leinenga *et al*., 2016). Based on these evidence, we incorporated transcranial ultrasonography in the RK Protocol. Thus, after having performed the first ART of step 1, a diagnostic ultrasound scan of the brain is performed as shown in Fig. 7. This figure shows the squama of the temporalis bone as a homogeneous hyperechoic structure; the meninges are visualized as a series of alternating hyper- and hypoechoic layers with a regular structure; the subarachnoid space appears as an anechoic line due to the presence of extra-axial fluid; and the gray matter of the temporal lobe (in this case, the right temporal lobe)

appears hypoechoic in comparison with the meninges showing a layered structure that corresponds to the cellular architecture of the cerebral cortex described by von Economo and Koskinas (1925). Study of the subarachnoid space may be used to evaluate the accumulation of extra-axial fluid according to the technique described in Bradstreet *et al*. (2014). However, at variance with our previous studies, at Sophia Health Institute, we do not limit our intervention to the evaluation of extra-axial fluid accumulation and how it relates to neurological symptoms, but we use that information to proceed with application of therapeutic ultrasounds to the brain and the deep cervical nodes (Fig. 2) with the intent of restoring the flow of cerebral lymph and possibly ameliorating neurologic symptoms when present (Bradstreet *et al*., 2015; Tarasoff-Conway *et al*., 2015).

In order to reach this goal, the deep cervical nodes that have been visualized by diagnostic ultrasonography are treated with therapeutic ultrasounds using a pulsed sequence indicated as 20% and a frequency of 3.3 MHz for 90 sec on each side of the neck. Only after having treated the nodes, the brain is treated with therapeutic ultrasounds that are administered through the temporal acoustic window using a pulsed sequence indicated as 10% and a frequency of 3.3 MHz for 90 sec on each side of the head. The rationale for this sequence of therapeutic procedures lays in the consideration that inflamed deep cervical nodes may pose an obstacle to the efflux of the lymph from the brain and, therefore, it is indicated to treat the deep cervical nodes first in order to reduce inflammation by exploiting the anti-inflammatory effects of pulsed ultrasounds (Jia *et al*., 2016). Subsequent treatment of the brain with therapeutic ultrasounds thus sends mechanical pressure waves consisting of alternate compression and relaxation that may favor the circulation of lymph in the brain lymphatic system and the removal of catabolites and toxins that may have become stagnant in the presence of an obstacle to the circulation of lymph (Raper *et al*., 2016). It is worth noticing that, given the anatomical proximity of the two structures (Fig. 2 and 3) application of therapeutic ultrasounds to the deep cervical nodes may correspond to treatment of the vagus nerve as well. Thus, it is well assessed that vagus nerve stimulation provides a number of benefits ranging from treatment of affective disorders in psychiatry (Cimpianu *et al*., 2017) to improvement in recovery from traumatic brain injury (Neren *et al*., 2016). Not surprisingly, the second ART, performed after each therapeutic ultrasound treatment, showed changes consistent with the effects described above.

Step 4

The fourth step of the RK Protocol consists in the second ART that is performed after application of therapeutic ultrasounds and in the comparison of the results with those obtained with the first ART of Step 1.

Step 5

The fifth step of the RK Protocol consists in the collection of the urine samples that has to be performed after the application of the therapeutic ultrasounds; the patient collects the naturally occurring next midstream urine in the sterile container provided by the laboratory performing the urine DNA test and the sample is shipped overnight to the laboratory. Although in this study we describe the results obtained with the urine DNA test, the presence of microbes, toxins, circulating cancer cells or other pathogenic noxae can be investigated in other biological matrixes such as stools, blood or serum or breath that are appropriately collected and analyzed by specialized laboratories.

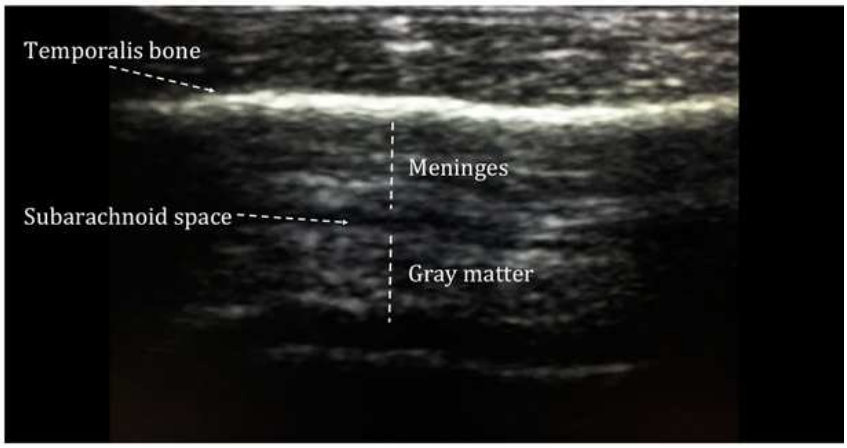


Fig. 7. Ultrasonographic appearance of the brain at the level of the right temporal lobe

Step 6

Successively, as the sixth step of the RK Protocol, the patient is treated with specific remedies that are targeted, for example, toward the pathogens identified with the previous steps. When antimicrobials are used, their choice is determined by applying common current understanding and fine tuning the selection with ART.

Step 7

The seventh step of the RK Protocol is performed in conjunction with the previous one and consists in the targeted application of therapeutic ultrasounds; such a procedure is performed once daily in the same manner described above in order to exploit the known therapeutic effects of pulsed ultrasounds that comprise anti-inflammatory effects, enhanced lymphatic drainage and optimization of drug uptake and utilization.

The RK Protocol is then repeated after three to four months in order to evaluate the effectiveness of treatment and to assess the treatment end-point.

Discussion

In this study we describe for the very first time a protocol, the RK Protocol, that is based on the integration of ART, as a screening tool and ultrasounds; the latter are utilized as a diagnostic tool, as a provocation tool and as a means to optimize drug uptake and utilization in specific areas of the body. This protocol consists of a precise sequence of diagnostic and therapeutic procedures with the ultimate goal of improving sensitivity and specificity of diagnosis and evaluating and optimizing efficacy of treatments in chronic conditions including, but not limited to, persistent Lyme. The sequence that we have developed is summarized in Table 1 and comprises, as a first step, a comprehensive ART screening, followed, as a second step, by total-body diagnostic ultrasonography that has the role of further refining the diagnostic hypotheses put forward by ART. Diagnostic ultrasonography also has the role to precisely identify the anatomic localization of the organs or the structures toward which the pressure waves of the therapeutic ultrasounds will then be directed in the third step of the protocol.

Application of therapeutic ultrasounds, the third step, plays three roles in the RK Protocol:

- It is used to exploit the well-known therapeutic effects of ultrasounds that have been observed in a variety of conditions associated with chronic inflammation or neurodegeneration (Jia *et al*., 2016; Leinenga *et al*., 2016)
- It is used to increase the potential effectiveness of remedies, an effect that can be further verified by the second, post-ultrasound ART (step 4). This phenomenon is due to the known efficacy of therapeutic ultrasounds to increase the uptake and utilization of remedies in cells and tissues (Bui *et al*., 2017)
- More specific to the protocol, it is used to force the exit of pathogens, toxins, transformed cells, cells infected by viruses and cells of the immune system that have interacted with all of the above, from tissue reservoirs or sanctuaries so to render them "visible" to the immune system and to the therapist who will then perform the fourth and fifth steps of this protocol

These are, respectively, the second ART screening performed after application of therapeutic ultrasounds (fourth step); and collection of samples for the molecular detection of microbes, toxins, circulating cancer cells etc. that can be detected in urine, stools, blood or serum, breath or other biological matrixes (fifth step).

We have observed that the results of the second ART, performed after application of therapeutic ultrasounds, are significantly different from those of the initial first ART. For example, signs that could be interpreted as the presence of pathogens associated with coinfections of persistent Lyme that had not been detected by the initial first ART exam, became apparent when the second ART was performed after application of therapeutic ultrasounds, thus improving the diagnostic sensitivity of the procedure. We noticed that also the inter- and intra-variability of ART results were significantly decreased when the test was performed after application of therapeutic ultrasounds.

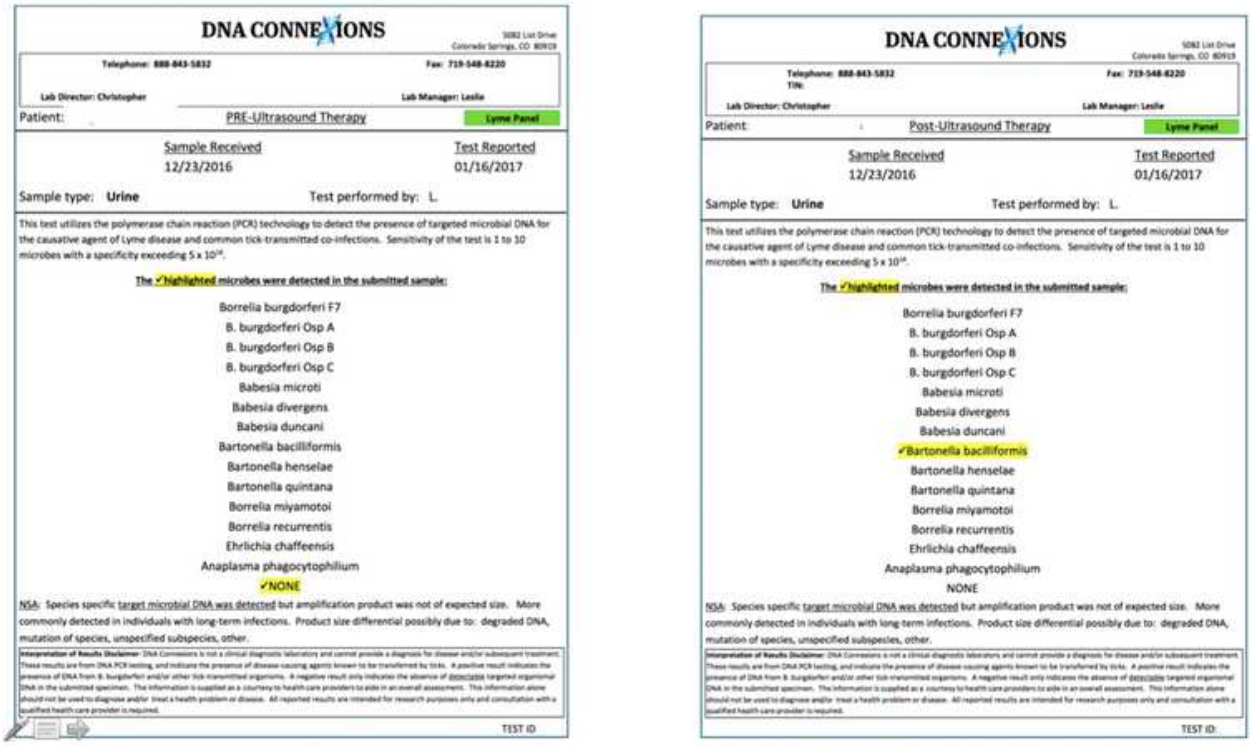
A major advantage of the RK Protocol consists in the possibility of validating the results obtained through the first steps using specific diagnostic methods that are based on molecular biology. In the case of persistent Lyme disease, for example, we used the urine DNA test that identifies genes pertaining to agents known to infect patients with persistent Lyme. Figure 8, shows a significant example of the results of the urine DNA test performed before and after application of therapeutic ultrasounds. The case of this patient with a history of angina that could not be causally diagnosed with the classic cardiology tests (such as stress echo etc.) is shown as a paradigmatic example representing a large majority of patients whom we have observed during the implementation of the RK Protocol at Sophia Health Institute. In this patient, urine samples were collected before and after application of therapeutic ultrasounds following the RK Protocol. In the urine sample collected before application of therapeutic ultrasounds (Fig. 8, left panel) the PCR-based DNA analysis did not reveal the presence of any pathogen among those detected by the Lyme panel even though the patient had tested positive for the presence of *Bartonella* species with ART. In

addition, this patient had tested negative for the presence of antibodies against *Bartonella henselae* (IgG/IgM) and negative also in the Western Blot test (CDC criteria) from the IgeneX lab in the blood draw obtained the same day before the application of the RK Protocol. In other words, before the application of therapeutic ultrasounds, this patient was one of the many cases where ART provided positive results that could not be confirmed by the urine DNA test or by antibody-based blood tests. However, the DNA test performed on the urine sample collected one hour after application of therapeutic ultrasounds, clearly shows the presence of one well-identified pathogen, *Bartonella bacilliformis*, a well-known cause of endocarditis, commonly diagnosed only post-mortem, whose presence, albeit suggested by ART, was not evident before the application of the ultrasounds (Fig. 8, right panel).

In proceeding with the implementation of the RK Protocol, the patient responded rapidly and favorably to targeted biological treatment involving daily therapeutic ultrasound application and several anti-microbial agents.

In our opinion, these results represent a paradigm change in diagnostics and therapy. Thus, the RK Protocol, enables to reach a clear diagnosis in cases that otherwise would have been labeled as "uncertain" or "undetermined" because of the discrepancy between ART and laboratory results. Quite obviously, a diagnosis based on the concordance of ART and laboratory results enables the therapist to implement specific therapeutic approaches that would have not been possible without the RK Protocol.

Although the RK Protocol was initially developed for persistent Lyme, its potential in other conditions ranging from cancer to neurodegenerative disorders did not escape our attention. For example, in the field of oncology the RK Protocol may serve as a tool for early diagnosis and as an instrument to increase the sensitivity of cancer cells to the therapeutic effects of a variety of approaches ranging from conventional radio- and chemotherapy to more recent forms of immunotherapy (Schwalb *et al*., 2016). In some aspects, the RK Protocol reminds the "shock and kill" strategy that is being pursued to eliminate HIV reservoirs that are responsible for the latency and persistence of the virus. This strategy aims at inducing HIV replication in latent viral reservoir; although this concept may appear counterintuitive since the goal of antiretroviral therapies should be to inhibit, not to stimulate, viral replication, the rationale is to make the virus "visible" to the immune system and to the antiretroviral drugs (Melkova *et al*., 2017). Analogously, the goal of the RK Protocol is to make pathogens, toxins, transformed cells and cells infected by viruses that are inaccessible to diagnostic and therapeutic tools, "visible" so that they can be recognized and dealt with, both by the therapist and the immune system.



DNA CONNEXIONS

Patient: PRE-Ultrasound Therapy — Lyme Panel

Sample Received 12/23/2016 — Test Reported 01/16/2017

Sample type: Urine — Test performed by: L.

This test utilizes the polymerase chain reaction (PCR) technology to detect the presence of targeted microbial DNA for the causative agent of Lyme disease and common tick-transmitted co-infections. Sensitivity of the test is 1 to 10 microbes with a specificity exceeding 5 x 10^{14}.

The ✓highlighted microbes were detected in the submitted sample:

Borrelia burgdorferi F7
B. burgdorferi Osp A
B. burgdorferi Osp B
B. burgdorferi Osp C
Babesia microti
Babesia divergens
Babesia duncani
Bartonella bacilliformis
Bartonella henselae
Bartonella quintana
Borrelia miyamotoi
Borrelia recurrentis
Ehrlichia chaffeensis
Anaplasma phagocytophilium
✓NONE

NSA: Species specific target microbial DNA was detected but amplification product was not of expected size. More commonly detected in individuals with long-term infections. Product size differential possibly due to: degraded DNA, mutation of species, unspecified subspecies, other.

TEST ID

DNA CONNEXIONS

Patient: Post-Ultrasound Therapy — Lyme Panel

Sample Received 12/23/2016 — Test Reported 01/16/2017

Sample type: Urine — Test performed by: L.

This test utilizes the polymerase chain reaction (PCR) technology to detect the presence of targeted microbial DNA for the causative agent of Lyme disease and common tick-transmitted co-infections. Sensitivity of the test is 1 to 10 microbes with a specificity exceeding 5 x 10^{14}.

The ✓highlighted microbes were detected in the submitted sample:

Borrelia burgdorferi F7
B. burgdorferi Osp A
B. burgdorferi Osp B
B. burgdorferi Osp C
Babesia microti
Babesia divergens
Babesia duncani
✓Bartonella bacilliformis
Bartonella henselae
Bartonella quintana
Borrelia miyamotoi
Borrelia recurrentis
Ehrlichia chaffeensis
Anaplasma phagocytophilium
NONE

NSA: Species specific target microbial DNA was detected but amplification product was not of expected size. More commonly detected in individuals with long-term infections. Product size differential possibly due to: degraded DNA, mutation of species, unspecified subspecies, other.

TEST ID:

Fig. 8. Example of urine DNA test for the detection of Lyme infection and co-infections

Thus, the RK protocol may find notable applications in the diagnosis of localized infections such as Lyme carditis (Robinson *et al*., 2015) or infections of the brain by *Babesia* (Aikawa *et al*., 1992). In these cases, that are notoriously difficult to diagnose, the RK Protocol has the additional benefit of allowing the localization of the infectious agents without resorting to invasive procedures. To this end, the therapeutic ultrasound treatment is applied to the areas of the body where the presence of the infectious agent is suspected and, if the diagnostic hypothesis is correct, the urine DNA test would show the presence of the pathogen only after application of therapeutic ultrasounds to specific areas. For example, if the presence of *Babesia* became evident only after application of therapeutic ultrasounds to the brain and not before, it could be concluded that the pathogen resided in the brain and was mobilized by the pressure waves generated by the therapeutic ultrasounds.

Therefore, the RK Protocol leads to profound new possibilities: Compartmentalized microbes, hidden transformed cells, toxins and metabolites could be detected using a safe and non-invasive method. In addition to these exciting possibilities in the field of diagnostics, the RK Protocol allows optimization of the therapeutic efficacy of drugs administered parenterally; thus, application of therapeutic ultrasounds before administration of drugs specifically targeted to microbes or toxins detected by the diagnostic steps of the protocol, would significantly increase uptake and utilization of the drugs themselves with a significant improvement of their therapeutic efficacy. ART would thus have the role of narrowing down the number of diagnostic hypotheses and therapeutic options with a resulting decrease of unnecessary, expensive and time-consuming tests.

Conclusion

We have developed a novel protocol for the non-invasive diagnosis and treatment of persistent Lyme and other chronic conditions due to persistent infections, toxicities, neoplastic transformation or neurodegeneration. The RK Protocol offers the advantage of being safe, rapid and relatively inexpensive and it can be easily implemented in any health institution whether in the developed world or elsewhere, without the need for sophisticated and expensive instruments. This protocol aims at achieving accurate and early diagnosis, at indicating the most appropriate therapeutic intervention and at maximizing the efficacy of specific therapeutic interventions.

Acknowledgement

The Authors wish to thank Ms. Daniela Deiosso for inspiring discussion and relentless support and the therapists at the Sophia Health Institute for their precious collaboration.

Author's Contributions

Dietrich Klinghardt: The inventor and developer of ART and performed all the diagnostic and therapeutic procedures described in this study.

Marco Ruggiero: Developed the ultrasound-based techniques described in this study, wrote the first draft of this paper, provided critical input and assisted in revising and improving the paper. He had no type of involvement in the treatment of patients.

Conflict of Interest

Dietrich Klinghardt is the inventor of ART and the founder of the Klinghardt Institute, the Klinghardt Academy, the Institut fuer Neurobiologie and the Sophia Health Institute, a private clinic. Dr. Klinghardt consults for several companies producing supplements and other remedies that, however, are not mentioned in this study. Marco Ruggiero is consultant for the company “dr. reinwald healthcare”, that provided the emulsion of chondroitin sulfate, vitamin D_3 and oleic acid mentioned in this study and he is the founder and CEO of the Swiss company Silver Spring Sagl, a company that produces supplements and probiotics. None of the products of Silver Spring Sagl is mentioned in this study. Marco Ruggiero is member of the Editorial Board of The American Journal of Immunology and is waived from the Article Processing fee for this contribution; he receives no remuneration for his editorial work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that the other author has read and approved the manuscript.

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